Part III

HO-1 and hepatobiliary injury after liver transplantation

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Expression of Heme oxygenase -1 in human livers before transplantation correlates with graft injury and function after transplantation

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Abstract

Upregulation of heme oxygenase-1 (HO-1) has been proposed as an adaptive mechanism protecting against ischemia/reperfusion (I/R) injury. We investigated HO-1 expression in 38 human liver transplants and correlated this with I/R injury and graft function. Before transplantation, median HO-1 mRNA levels were 3.4-fold higher (range 0.7-9.3) than in normal controls. Based on the median value, livers were divided into two groups: low and high HO-1 expression. These groups had similar donor characteristics, donor serum transaminases, cold ischemia time, HSP-70 expression, and distribution of HO-1 promoter polymorphism. After reperfusion, HO-1 expression increased significantly further in the initial low HO-1 expression group, but not in the high HO-1 group. Postoperatively, serum transaminases were significantly lower and bile salt secretion was higher in the initial low HO-1 group, compared to high expression group. Immunofluorescence staining identified Kupffer cells as the main localization of HO-1.

In conclusion, human livers with initial low HO-1 expression (< 3.4 times controls) are able to induce HO-1 further during reperfusion and this is associated with less injury and better function than initial high HO-1 expression (> 3.4 times controls). These data suggest that increase in HO-1 during transplantation is more protective than a high HO-1 expression before transplantation.

Introduction

Orthotopic liver transplantation (OLT) is an effective treatment for end-stage liver diseases (1). However, ischemia and subsequent reperfusion of the liver remain a major cause of graft injury, causing liver dysfunction and even failure after transplantation (2). This is particularly true for livers from older donors and steatotic livers, which have a higher susceptibility to ischemia/reperfusion (I/R) injury (3,4). During organ procurement and transplantation, the liver is exposed to oxidative stress. Besides the ischemia during cold storage, hypoxia may occur before or during procurement due to hypotension or cardiac arrest in the donor. After graft reperfusion, several cascades are triggered leading to the formation of reactive oxygen species (ROS), which are well-known sources of oxidative stress. Methods to protect liver grafts against I/R injury have considerable clinical consequences and are therefore of great interest.

It is increasingly recognized that cells respond to stressful events, such as ischemia, hypoxia and ROS, by the activation of various cytoprotective genes and pathways. Heme oxygenase-1 (HO-1) has recently been proposed as a graft survival gene (5,6). Up-regulation of HO-1 is considered to be one of the most critical cellular protection mechanisms (7,8). It is rapidly induced under various conditions of oxidative stress, including hypoxia, hyperoxia and ROS (9). HO-1 catalyzes the rate-limiting step in the oxidative detoxification of excess heme, by cleaving the α-methene bridge into equimolar amounts of free iron, biliverdin and carbon monoxide (CO) (9). Free iron, catalyzing oxidative reactions, is bound by iron regulatory proteins that stimulate synthesis of ferritin, thereby preventing iron-dependent oxidative stress (10,11). Biliverdin is subsequently converted into bilirubin and both have the ability to scavenge ROS (12-15). CO has been shown to serve as an endogenous regulator for maintaining microvascular blood flow of the liver (16,17).

Two- to three-fold induction of HO-1 by pharmacologic agents or genetic engineering has been shown to reduce I/R injury in rat liver grafts after extended cold ischemia time (6). Moreover, steatotic livers from genetically obese Zucker rats are markedly protected against I/R injury after exogenous upregulation of HO-1 (5). Based on these observations, exogenous induction of HO-1 prior to transplantation has been proposed as a potentially powerful therapeutic option to protect liver grafts against I/R injury (5,6). Molecules such as HO-1, however, are probably not exclusively cytoprotective and each of the products generated by the action of heme oxygenase (Fe^{2+}, bilirubin and CO) can cause injury under certain circumstances (18). Indeed,

several experimental studies have shown that excessive overexpression of HO-1 is directly related with increased injury (19-21). Recently, also a $(GT)_n$ dinucleotide repeat polymorphism that modulates the level of HO-1 inducibility was identified in the promoter region of the human HO-1 gene. Short GT repeats (<25) are associated with highly significant upregulation of HO-1 in response to inflammatory stimuli (22,23). Therefore, it is critically important to understand the endogenous changes in HO-1 expression under clinical conditions, such as transplantation, before the exogenous induction of HO-1 can be safely attempted as a possible therapeutic or prophylactic measure to reduce I/R injury.

We have therefore studied the changes in endogenous HO-1 expression in human liver grafts before and after transplantation and correlated these with biochemical markers of graft injury and hepatobiliary function. This study provides important new information on the role of endogenous HO-1 expression during human liver transplantation

Patients and Methods

Patient and Donor Data

Thirty-eight patients undergoing OLT were included. All patients received livers from brain death, multi-organ donors. In the control group (n=5), biopsies were collected in patients undergoing partial hepatectomy for metastatic tumors. Tissue and data collection was performed according to the guidelines of the medical ethical committee of our institution and the Dutch Federation of Scientific Societies.

Collection of Liver Biopsies and Bile Samples from Recipients

Three sequential needle biopsies were taken from each liver graft: at the end of cold storage, 3 hours after reperfusion and 1 week after transplantation. Biopsies were immediately divided: one part was snap-frozen in liquid nitrogen for RNA and protein isolation and one part was frozen in isopentane at -80°C for histology studies. During transplantation a bile drain was routinely placed into the common bile duct, allowing collection of bile (24). To avoid interruption of the entero-hepatic circulation bile was daily readministered via a jejunostomy catheter. After the transplantation, bile samples were collected daily between 8 and 9 am. Liver and bile specimens were stored at -80°C.

RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was isolated from liver biopsies using TRIzol (Invitrogen Life Technologies, Breda, the Netherlands) and quantified using Ribogreen (Molecular Probes, Inc., Eugene, OR). Reverse transcription was performed on 3.36 µg RNA using random primers in a final volume of 75 µl (Reverse Transcription System, Promega, Madison, WI). For quantitative real-time detection RT-PCR (25,26), sense and antisense primers (Invitrogen, Paisley, Scotland) and fluorogenic probes (Eurogentec, Herstal, Belgium) for HO-1, HSP-70 and 18S were designed using Primer Express software (PE Applied Biosystems, Foster City, CA). For HO-1, the primers and probe used were 5'-GACTGCGTTCCTGCTCAACAT-3' (sense), 5'-GCTCTGGTCCTTGGTGTCATG-3' (antisense), and 5'-TCAGCAGCTCCTGCAACTCCTCAAAGAG-3' (probe), generating a 75 base pair PCR product. For heat shock protein-70 (HSP-70), used as a molecular stress marker, the following primers and probe were used: 5'-TCTTCTCGCGGATCCAGTCT-3' (sense), 5'-GGTTCCCTGCTCTCTGTCG-3' (antisense), and 5'-CCGTTTCCAGCCCCCAATCTCAG-3' (probe), generating a 70 base pair PCR product. For 18S, the primers and probe used were 5'-CGGCTACCACATCCAAGG-3' (sense), 5'-CCAATTACAGGGCCTCGAAA-3' (antisense), and 5'-CGCGCAAATTACCCACTCCCGA-3' (probe), generating a 109-base pair PCR fragment. The ABI PRISM 7700 (Applied Biosystems, Foster City, CA) was used for PCR.

Protein Isolation and Western Blot Analysis

Frozen liver tissue was homogenized in buffer containing protease inhibitors. Protein concentrations were measured using a standard Lowry assay. Fifteen µg of protein was fractioned on a 5% SDS-PAGE gel and transferred to PVDF membranes (Pall Life Sciences, Ann Arbor, MI). The membranes were blocked with 1% SKIM milk (Fluka BioChemica, Buchs, Switzerland) and labeled with the anti HO-1 polyclonal antibody (dilution, 1:5000, StressGen, Victoria, British Columbia, Canada). After washing in PBS/0.05% Tween-20 (Sigma, Malden, The Netherlands), blots were incubated with a horseradish peroxidase-labled goat anti-rabbit IgG (dilution, 1:2000, DAKO, Glostrup, Denmark). Finally membranes were developed with ECL (Amersham, Chalfont St Giles, UK). Five separate cases were examined in each group.

HO-1 Genotype assessment

Genomic DNA was isolated from donor splenocytes using a commercial kit (Gentra Systems, Minneapolis, MN). PCR and genotyping procedures were similar as described by de Jong et al. (27). The 5'-flanking region of the HO-1 gene containing the poly $(GT)_n$ repeat was amplified by PCR using as forward primer 5'-CAGCTTTCTGGAACCTTCTGG-3', carrying a 6-FAM flourescent label (Sigma, Malden, the Netherlands), and as reversed primer 5'-GAAACAAAGTCTGGCCATAGGAC-3'. Sequence analysis of the amplification products of individuals homozygous for the 222 and 229 basepairs alleles showed correspondence with GT numbers 26 and 29, respectively (results not shown). We divided allelic repeats into two subclasses using a classification as previously described in transfection studies (28). Short repeats, with less than 25 GT repeats (amplicons of 220 basepairs and less), were designated as allele class S (short), and long repeats with 25 or more GT repeats as allele class L (long). Recipients of class S allele liver transplants (homozygous S/S and heterozygous S/L) were compared with recipients of non-class S allele transplants (L/L).

Immunofluorescence Microscopy

Frozen liver sections were stained for HO-1 and the Kupffer cell marker CD68, using an anti-HO-1 polyclonal antibody (dilution, 1:100, StressGen) and an anti-human CD68 monoclonal antibody KP-1 (dilution, 1:2000, DAKO). After washing, sections were subsequently incubated with a goat anti-rabbit IgG with a red fluorescent label (Alexa Fluor 568, Molecular Probes, Leiden, the Netherlands), and with a goat anti-mouse IgG with a green fluorescent label (Alexa Fluor 488, Molecular Probes). Double-positive cells were identified as those stained yellow. Percentages of HO-1-positive Kupffer cells were calculated by dividing the number of cells stained yellow by the number of cells stained green (29). Five different high power fields (x400) were analyzed in an individual liver sample, and five separate cases were examined in each group. Images were taken with a Leica DM LB fluorescence microscope (Leica, Wetzlar, Germany).

Total Bile Salt Secretion and Serum Biochemistry

Postoperatively, bile flow was expressed as daily bile production in mL per kg body weight of the donor. Total bile salt concentration was measured spectrophotometrically with 3α-hydroxysteroid dehydrogenase (30). Serum samples were analyzed for aspartate- and alanine aminotransferase (AST and ALT) and gamma glutamyltransferase (GGT), by routine clinical chemistry testing.

Statistics

Statistical analyses were performed using SPSS Version 11.5 for Windows (SPSS Inc., Chicago, IL). All data are reported as median and interquartile ranges (IQR). Groups were compared with the Mann-Whitney U-tests, Wilcoxon Signed Ranks-tests, Pearson X2-tests and the Fisher's Exact Test where appropriate. Postoperative biochemical variables were compared using the daily values, but also the total course during the first week was compared by calculating the area under the curve (AUC), using the trapezium rule. All P values were 2-tailed and considered as statistically significant at levels < 0.05.

Results

Effects of OLT on HO-1 Gene and Protein Expression.

Before transplantation, the median HO-1 mRNA level was 3.4-times higher in donor livers than in normal control livers (P = 0.001; Figure 1), suggesting that HO-1 is already induced in brain-death donors or during organ procurement.

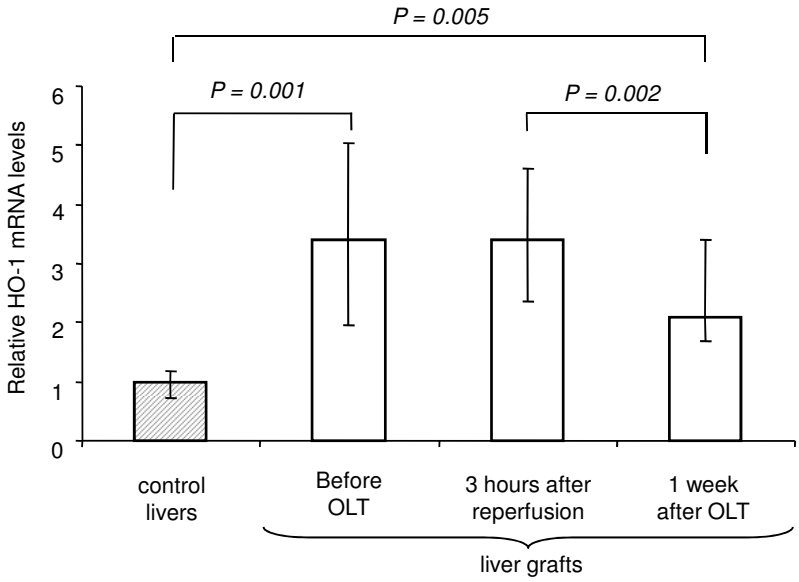


Figure 1. HO-1 mRNA levels in human liver grafts (n=38) and normal control livers (n=5). HO-1 mRNA was standardized for 18S rRNA. HO-1 expression in control livers was set to 1.0. Values represent medians and interquartile ranges.

At 3 hours after reperfusion, there was no significant overall change in HO-1 expression. One week after transplantation, HO-1 gene expression decreased by 38% compared to the values after reperfusion (P = 0.002; Figure 1). However, HO-1 expression remained strongly elevated during the first postoperative week compared to normal control livers (Figure 1).

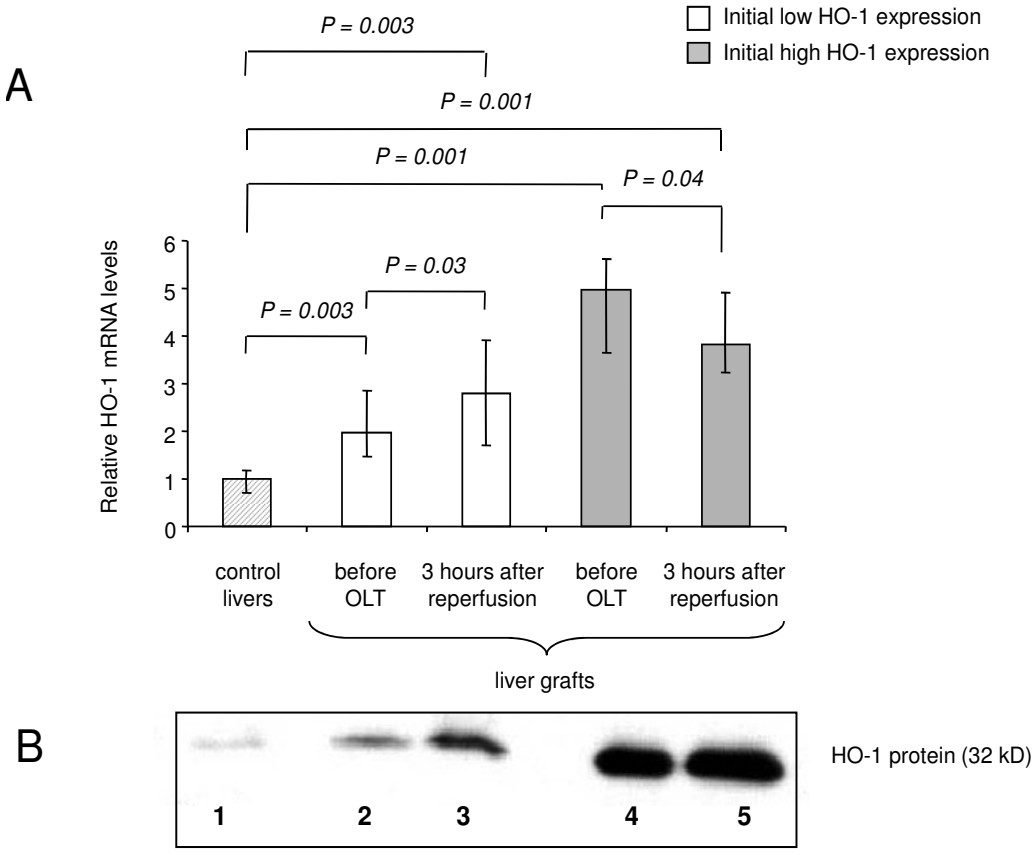


Figure 2. A) Course of HO-1 mRNA levels in human liver grafts with low or high HO-1 expression before transplantation; initial low HO-1 expression group (n=19) and initial high HO-1 expression group (n=19), respectively. HO-1 mRNA was standardized for 18S rRNA. HO-1 mRNA levels in normal control livers was set to 1.0. Values represent medians and interquartile ranges. B) Western blot analysis of HO-1 protein expression in the initial low and initial high HO-1 group.

A wide variation in HO-1 gene expression was detected in liver biopsies that were collected before transplantation, ranging from 0.7- to 9.3-times the levels in normal control livers. To be able to identify donor variables that are associated with HO-1 induction, and to study the possible impact of HO-1 on I/R injury and graft viability after transplantation, we decided to divide liver grafts into two groups based on the level of HO-1 expression before transplantation. A low HO-1 expression group (n=19) was formed by livers with an initial HO-1 mRNA level below the median value (< 3.4-times control levels) and a high HO-1 expression group (n=19) was formed by livers with an initial HO-1 gene expression above the median value (> 3.4-times control levels). Median HO-1 expression in the low and high expression group was 2.0- and 5.0-times higher than in control livers (Figure 2A). Interestingly, HO-1 mRNA level increased significantly by 43% after reperfusion in the initial low expression group, whereas HO-1 expression decreased by 23% after reperfusion in the inital high expression group (Figure 2A). In both groups, HO-1 gene expression remained significantly elevated during the first postoperative week, compared to controls (data not shown).

Changes in HO-1 protein concentrations, as detected by Western blot analysis, were similar to the changes in HO-1 mRNA expression. HO-1 protein concentration was low in normal control livers, compared to the donor livers. After reperfusion, HO-1 protein expression increased further in the initial low HO-1 expression group, but not in the initial high HO-1 group (Figure 2B).

Comparison of Donor Data for Livers with Low and High HO-1 Expression

A large number of donor characteristics and laboratory values were investigated in an attempt to explain the differences in HO-1 gene expression before transplantation. Several events that are known to induce HO-1 expression in animal models, such as hypotension, cardiac arrest, blood transfusions and ischemia, may also occur in brain-death donors or during organ procurement. In addition to this, some drugs (i.e. dopamine) have been shown to induce HO-1 expression (31). We have compared all these donor-related events and variables in the two groups, but were unable to find statistically significant differences (Table 1).

Table 1. Comparison of donor, recipient and surgical variables in initial low HO-1 expression group and initial high HO-1 expression group.

	Low HO-1 Expression		High HO-1 Expression	
Donor variables				
Age (years; median [IQR])	39	(25-60)	48	(41-58)
Gender (M/F)	7/12		8/11	
ICU stay (days; median [IQR])	2.5	(0.8-4.5)	1.2	(0.3-3.2)
Duration of liver procurement (minutes; median [IQR])	150	(51-177)	150	(67-195)
Hypotension (no. of donors)a	7/19		11/19	
Cardiac arrest (no. of donors)b	2/19		3/19	
Dopamine (no. of donors)c	8/19		11/19	
Bloodtransfusion (no. of donors)c	5/19		7/19	
Temperature (oC; median [IQR])	36.1	(36.0-36.8)	36.5	(36.1-37.0)
Diuresis last hour (ml; median [IQR])	220	(113-300)	200	(130-320)
Bloodpressure (mmHg; median [IQR])	120/60	(110/60-124/73)	120/67	(110/65-137/78)
pO2 (kPa; median [IQR])	16.5	(13.1-21.8)	13.6	(11.8-20.1)
FiO2 (%; median [IQR])	40	(36-47)	40	(40-57)
AST (U.L-1; median [IQR])	27	(15-93)	42	(19-67)
ALT (U.L-1; median [IQR])	24	(18-61)	25	(14-45)
GGT (U.L-1; median [IQR])	20	(15-29)	20	(13-63)
Total Bilirubin (U.L-1; median [IQR])	4.0	(1.3-10.0)	10.0	(5.0-16.5)
Hemoglobin (mmol.L-1; median [IQR])	7.6	(6.3-8.9)	7.0	(5.8-8.9)
Recipient and Surgical variables				
Age (years; median [IQR])	45	(28-58)	47	(35-54)
Gender (M/F)	9/10		13/6	
ICU stay (days; median [IQR])	3	(2-6)	2	(2-7)
Acute rejection of the graft (no. of recipients)d	11/19		4/19	
1st Warm Ischemia Time, WIT (minutes; median [IQR])e	43	(36-57)	42	(28-49)
Cold Ischemia Time, CIT (minutes; median [IQR])	465	(415-567)	574	(457-620)
2nd WIT (minutes; median [IQR])f	43	(37-47)	48	(43-56)

a) Donors who suffered at least one episode of hypotension or b) cardiac arrest within 24 hrs prior to procurement of the liver.

c) Number of donors who were administered dopamine or blood within 24 hrs before donor hepatectomy.

d) Number of recipients who suffered from rejection of the graft within the first week after transplantation.

e) 1st WIT: time between start cold perfusion in the donor and procurement of the liver graft.

f) 2nd WIT: time between the end of cold ischemic preservation of the liver and start of reperfusion in the recipient.

There were no statistical significant differences for any variables between the two groups (Mann Whitney U-test or Pearson Chi-Square-test).

There were also no significant differences in the time between start of in situ cold perfusion in the donor and actual hepatectomy (1st “relatively” warm ischemia) or in the duration of cold storage (Table 1). Interestingly, there were also no differences in donor serum markers of liver injury (AST, ALT and GGT) or liver function (bilirubin) between the two groups (Table 1). Moreover, there was no significant difference in pretransplant mRNA expression of the stress protein HSP-70 in the low and high HO-1 group (1.18 [IQR 0.30 – 3.76] versus 0.57 [IQR 0.22 – 2.27]; p = 0.44). These data suggest that differences in HO-1 expression in liver grafts before transplantation cannot simply be explained by a higher number of compromised donors in the high HO-1 expression group.

The Effect of HO-1 Donor Genotype

To examine whether the differences in initial HO-1 expression could be explained by the the number of $(GT)_n$ repeats in the HO-1 promoter region, HO-1 donor genotypes were analyzed. Allele class S/S was present in 8% of the donors, 35% of the donors were heterozygous for class S alleles (S/L), and 57% of the donors were non-carriers of the class S allele (L/L). Distribution of the numbers of $(GT)_n$ repeats was not different for donor livers in the initial low and high HO-1 expression group (Figure 3). There were also no significant differences in the distribution of class S allele donor livers (S/S and S/L) and non-class S donor livers (L/L) in the two groups (Table 2).

Table 2. Distribution of HO-1 genotype in the livers with initial low or high HO-1 mRNA expression.

	Initial HO-1 Expression		
Genotype*	**Low**	**High**	
Short Allele (SS or SL)	8 (42%)	8 (44%)	p-value = 1
Long Allele (LL)	11 (58%)	10 (56%)	
	19 (100%)	18** (100%)	

a) Short allele (S) status defined as < 25 (GT) repeats in the HO-1 promoter region; Long allele (L) status defined as > 25 (GT) repeats in the HO-1 promter region.

b) Genomic DNA for gene sequencing was not available in one donor.

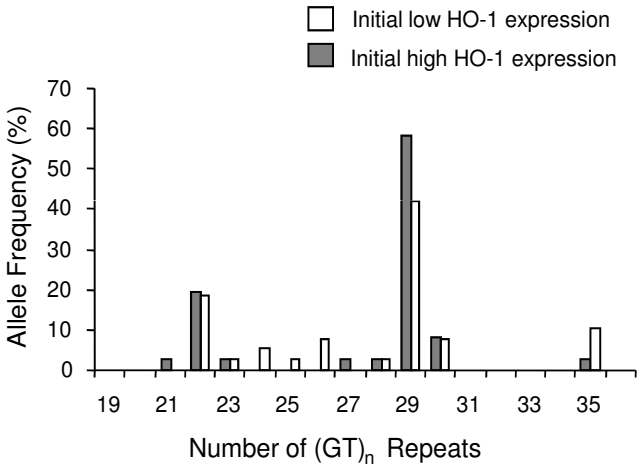


Figure 3. Allele frequencies of the HO-1 $(GT)_n$ repeat promoter polymorphism in liver grafts with initial low or high HO-1 mRNA expression.

Post-transplant Outcome in Relation to HO-1 Expression

To examine whether the magnitude of HO-1 induction was associated with differences in outcome after transplantation, laboratory values and recipient characteristics were analyzed. Posttransplant serum levels of AST and ALT were used as well-accepted markers of I/R injury. Although there were no differences in serum AST levels in the donors, we found a significant positive correlation between serum AST levels in the recipient on postoperative day 1 and HO-1 expression in the donor liver before transplantation (Figure 4). When comparing the two groups, serum AST levels on postoperative days 1 through 3 were significantly higher in recipients of livers with high HO-1 expression (Figure 5A). Also serum ALT levels were significantly higher on postoperative day 1 in recipients of livers with high HO-1 expression (Figure 5B). Hepatobiliary function, as reflected by biliary bile salt secretion, was significantly worse in the group with high HO-1 expression, compared to the group with low expression (Figure 5C). When groups were categorized based on the ability of increasing HO-1 expression during reperfusion of the liver graft, serum AST levels in the induction group (n=15) were significantly lower on postoperative days 2 and 3 than in the HO-1 reduction group (n=23). Serum ALT levels and biliary bile salt secretion however, did nof differ between the groups in the latter classification (data not shown).

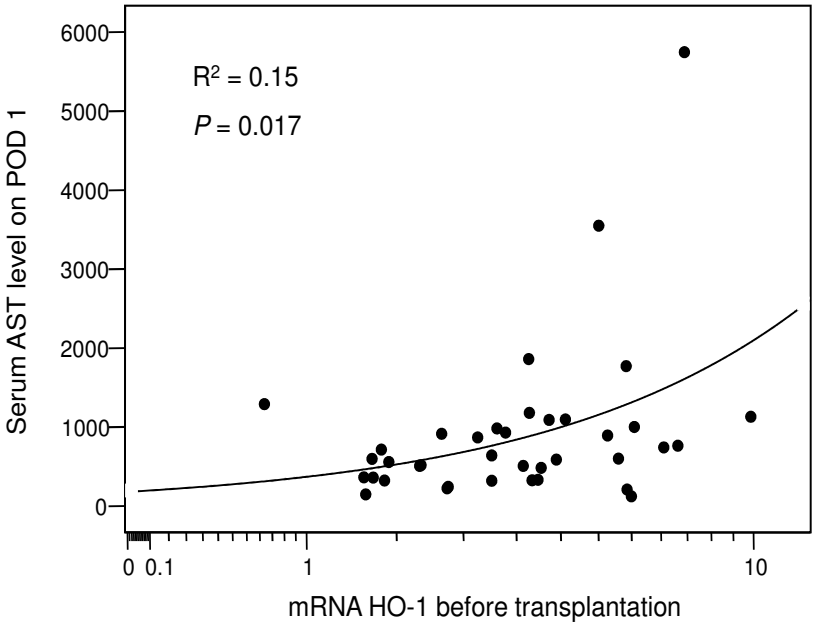


Figure 4. Correlation between hepatic HO-1 mRNA expression before transplantation and serum AST level on postoperative day 1 (POD 1) in all liver transplant recipients (n=38).

These findings indicate that liver grafts with an initial high (> 3.4-fold) HO-1 expression before transplantation exhibited more I/R injury and have poorer hepatobiliary function after transplantation than grafts with an initial low (< 3.4-fold) HO-1 expression, despite the fact that there were no differences in biochemical or molecular markers of graft injury in the donor before organ procurement.

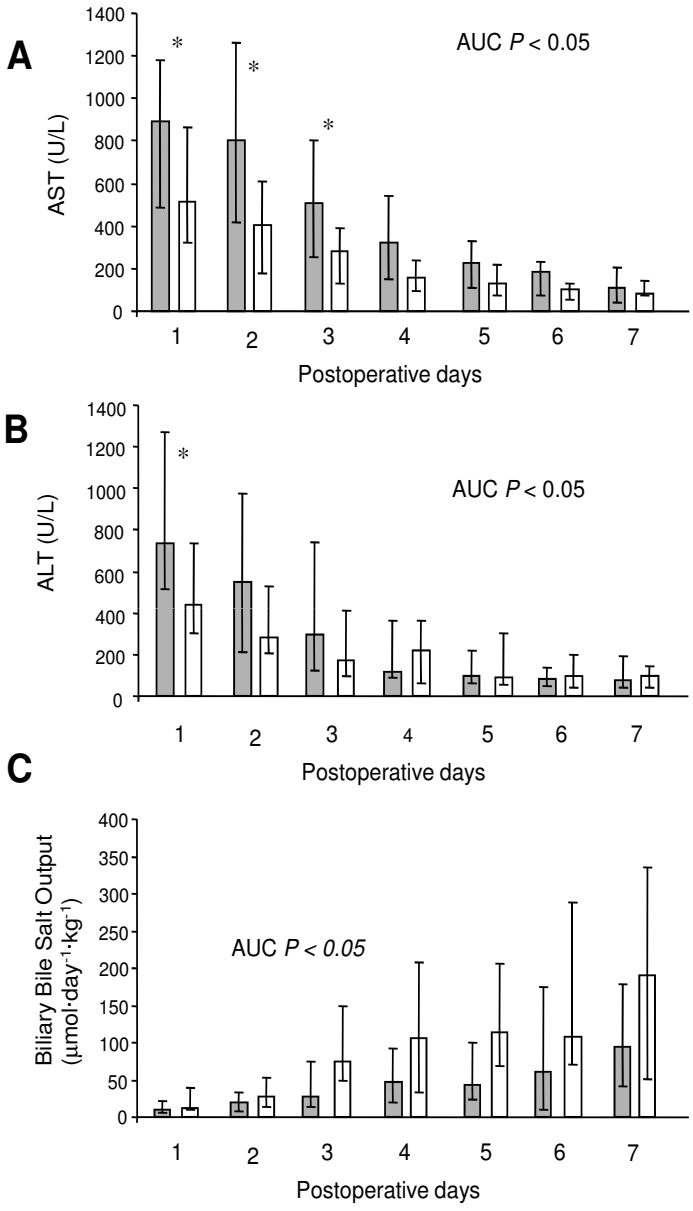


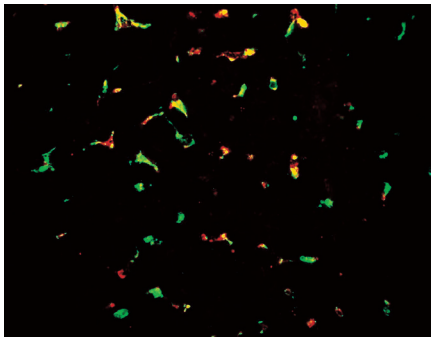
Figure 5. Serum AST (panel A) and ALT (panel B) levels and biliary bile salt secretion (panel C) in the first week after OLT in the initial low (open bars; n=19) and high HO-1 (closed bars; n=19) expression groups. Values represent medians and interquartile ranges. The asterisks indicate significant differences between the groups (p<0.05). Total course during the first week was calculated as the area under the curve (AUC), using the trapezium rule.

Immunofluorescence Microscopy

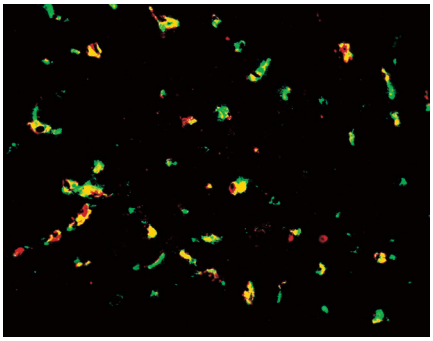
Specific immunostaining showed that HO-1 was predominantly localized in irregular and star-shaped cells. These characteristics suggested that HO-1 protein is mainly expressed in Kupffer cells, which was confirmed by double-color immunofluorescence labeling, using the anti-HO-1 and anti-human CD68 MoAb KP-1, a marker of Kupffer cells. As shown in Figure 6, the distribution of anti-HO-1 positive (red) cells overlapped with that of KP-1-positive (green) cells, resulting in a yellow staining. In control livers, a considerable proportion of Kupffer cells did not express HO-1-associated immunoreactivity and displayed mainly a green staining (Figure 6A). In contrast with this, almost all Kupffer cells in liver grafts demonstrated positive staining for HO-1 (Figure 6B-E). Indeed, morphometrical analysis showed significantly higher percentages of HO-1-positive Kupffer cells in liver grafts before transplantation, compared to normal control livers (low and high HO-1 expression group 88% and 95%, respectively, compared to 50% in normal control livers, $P < 0.02$ for both groups; Table 3). After reperfusion, HO-1 expression in Kupffer cells increased further, resulting in a positive staining of all Kupffer cells in both groups (Table 3).

Although, after reperfusion, all Kupffer cells in both groups stained positive for HO-1, the red staining (HO-1) per cell was far more intense in the group with high HO-1 expression than in the low expression group (Figure 6C and E). This indicates that not only the percentage of Kupffer cells expressing HO-1 is increased in liver grafts, but that also the HO-1 protein expression per Kupffer cell is enhanced, where the latter seems to discriminate the group with high HO-1 expression from the livers with low HO-1 expression. This is in line with the higher HO-1 mRNA and protein levels after reperfusion in the group with high HO-1 expression, compared to the low expression group.

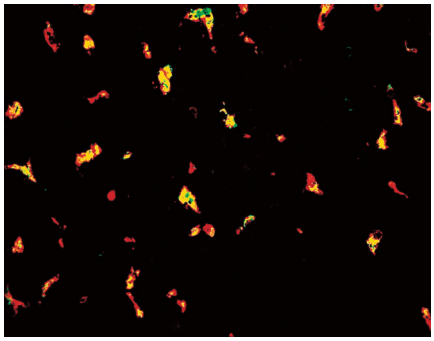
A. Normal control liver



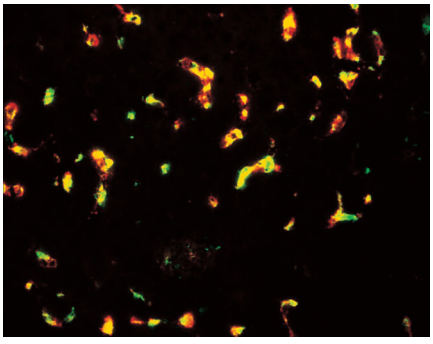
B. Initial low HO-1 expression: before OLT



C. Initial low HO-1 expression: after reperfusion



D. Initial high HO-1 expression: before OLT



E. Initial high HO-1 expression: after reperfusion

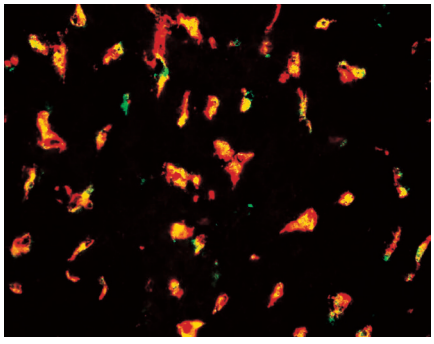


Figure 6. Immunofluorescence double-staining of liver biopsies. Sections are stained for HO-1 (red) and the Kupffer cell marker CD68 (green). Colocalization of these two colours can be recognized by the yellow colour. Panel A; normal control liver. Panel B; pretransplant biopsy of a liver with low initial HO-1 mRNA expression. Panel C; postreperfusion (3hrs) biopsy of a liver with low initial HO-1 mRNA expression. Panel D; pretransplant biopsy of a liver with high initial HO-1 mRNA expression. Panel E; postreperfusion biopsy (3hrs) of a liver with high initial HO-1 mRNA expression.

Table 3. Morphometrical analysis of cell type specific expression of HO-1 in human liver transplants with low or high HO-1 expression and control livers.

	Control	Initial Low HO-1 Expression		Initial High HO-1 Expression	
		Before OLT	After Reperfusion	Before OLT	After Reperfusion
Single immunostaining					
HO-1(+) (no. of cells; median [IQR])[a]	20 [17-23]	31 [27-47][e]	27 [17-36][e]	40 [37-44][e,f]	37 [27-44][e]
CD68(+) (no. of cells; median [IQR])[b]	37 [35-43]	31 [25-40]	30 [23-36]	42 [40-44][f]	36 [33-43][g]
Double immunostaining					
HO-1(+) Kupffer cells (no. of cells; median [IQR])[c]	20 [17-23]	28 [23-35]	23 [13-30]	40 [37-43][f]	35 [30-43][g]
% HO-1(+) Kupffer cells (%; median [IQR])[d]	50 [45-63]	88 [78-99][e]	100 [40-100][e]	95 [93-100][e]	100 [88-100][e]

a) Number of HO-1 and b) CD68 positive cells.
c) Number and d) percentage of HO-1 positive Kupffer cells.
Analyses based on observations in five different high power fields within one liver biopsy at 400X
e) $P < 0.02$, compared with the control group
f) $P < 0.03$, compared with the values before OLT of the initial low expression group
g) $P < 0.01$, compared with the values after reperfusion of the initial low expression group

Discussion

We have investigated HO-1 expression in human liver allografts during transplantation and correlated this with clinical signs of graft injury and hepatobiliary function. There are three novel findings in this study. First, we have shown that, compared to normal control livers, HO-1 gene and protein expression in human liver grafts from brain-death donors is induced already prior to transplantation. After reperfusion, HO-1 expression increased further in livers with relatively low initial HO-1 expression (< 3.4 times controls), but not in livers with initial high HO-1 expression (> 3.4 times controls). Second, allografts with initial high expression of HO-1 demonstrated significantly more I/R injury and had worse hepatobiliary function than grafts with a low upregulation of HO-1. Finally, we were able to identify Kupffer cells as the main site of HO-1 protein expression in human liver grafts. While about 50% of the Kupffer cells in normal control liver expressed HO-1, positive staining for HO-1 was found in 100% of the Kupffer cells of transplanted livers. These findings provide important new information on the endogenous regulation of HO-1 during human liver transplantation.

There is accumulating evidence that the HO-1 system has important vasoregulatory properties and actively maintains hepatic microperfusion and tissue oxygenation via the production of CO (16). In addition to this, the HO-1 system has been shown to have anti-oxidant, anti-inflammatory, anti-apoptotic and platelet aggregation-inhibiting properties and, therefore, it has been proposed a graft survival gene. Animal studies have suggested that exogenous induction of HO-1 before transplantation may confer cytoprotective and immune regulatory functions (6,32-34) and could become a novel and potentially powerful strategy to protect (marginal) liver grafts from I/R injury (5,8). Induction of HO-1 can be obtained by a variety of methods, such as administration of HO-1 inducers (i.e. cobalt protoporphyrin) or adenoviral HO-1 genetransfer (5,8). These methods generally lead to a 2 to 3-fold upregulation of HO-1 activity (5). There is increasing evidence that overexpression of HO-1 higher than this is not exclusively cytoprotective (19,21). In fibroblast cell cultures, low induction of HO-1 (less than 5-fold) was shown to be cytoprotective against hyperoxia, but excessive HO-1 activation resulted in the accumulation of free divalent iron and increased oxidative injury (19). Moreover, it has been shown that highly increased (about eight- to nine-fold) activity of HO-1 contributes to endotoxin-induced shock in rats, due to the increased production of CO, a potent vasorelaxant (21). Therefore, it is of paramount importance that the endogenous changes in

HO-1 expression during transplantation, as well as the therapeutic window of protection, are well defined before clinical application of HO-1 inducing protocols are attempted.

All donor livers in our study were obtained from brain-death multi-organ donors. The increased HO-1 mRNA and protein expression observed in these livers before transplantation suggests that HO-1 is induced in brain-death donors. This observation is in line with studies in kidney allografts from brain-death donors (35). We have tried to identify variables which could have contributed to the increased expression of HO-1 in the donor livers before transplantation. Several factors have been shown to induce HO-1 gene expression in vivo, including hypotension (36), hypoxia (37-39), hyperoxia (9,40), blood transfusions (41,42), and inotropic drugs like dopamine (31). All of these factors may also occur in postmortem organ donors. Comparison of these known inducers of HO-1 gene expression, as well as several other donor and procurement related variables, however, did not show any statistically significant differences between the two groups. Variations in initial HO-1 expression could also not be explained by differences in the distribution of the $(GT)_n$ repeat polymorphism of the HO-1 promoter. The functionally relevant short allele status (<25 repeats) was not found more frequently in livers with initial low HO-1 expression. Further studies will be necessary to elucidate the mechanisms of endogenous HO-1 induction in organs from brain death donors.

Although we did not find differences in biochemical (liver enzymes) or molecular (HSP-70) markers of liver injury before transplantation between the liver grafts with low or high HO-1 expression, we did observe a significant correlation between postoperative serum AST in the recipients and initial HO-1 expression. In parallel with this, serum AST levels were significantly higher and biliary bile salt output significantly lower after transplantation in recipients of livers with high HO-1 expression, compared to grafts with low HO-1 expression. Liberation of divalent iron is one of the effects resulting from increased HO-1 activity (9). Iron is a mediator of the generation of ROS and it has been shown to play an important role in I/R injury (43,44). We, therefore, speculate that exaggerated HO-1 activity in liver grafts may cause increased injury due to the liberation of iron, resulting in a pro-oxidant condition and higher susceptibility to I/R injury. The apparent paradox of one molecule or pathway causing both cytoprotection and cytotoxicicty has also been found in other systems, like the nitric oxide system (45). More studies will be needed to clarify this issue.

Interestingly, a significant further increase in HO-1 expression was found after reperfusion of livers with an initially low expression, whereas a small, but significant decrease in HO-1 expression was

observed in livers with initially high HO-1 expression. This data could imply that HO-1 mRNA expression cannot be further upregulated upon reperfusion when levels are already high to start with, whereas further upregulation can occur in livers with moderately elevated HO-1 expression before reperfusion. Although we observed a better postoperative outcome in the initial low HO-1 expression group, it remains indefinite whether it is the initial low HO-1 expression or the ability to increase HO-1 expression upon reperfusion that confers cytoprotection.

We identified Kupffer cells as the main site of HO-1 expression in human livers. Makino et al. (29) have recently reported similar findings in human cirrhotic livers. These studies in human liver are in contrast with data from rat livers, where considerable expression of HO-1 has also been found in hepatocytes (46). While in our study about 50% of the Kupffer cells in the control livers expressed HO-1, this was more than 80% in the liver grafts before transplantation and even 100% after transplantation. These findings suggest that a subpopulation of Kupffer cells, which does not express HO-1 under normal circumstances may induce HO-1 expression. It has been suggested that Kupffer cells may serve as sensor cells detecting local hemodynamic changes and mechanical forces in sinusoids (29,47). By increasing HO-1 activity and the generation of the vasorelaxing gaseous CO, Kupffer cells are able to maintain microvascular blood flow in the liver (29). On the other hand, it is well-known that Kuppfer cells play a critical role in the pathogenesis of I/R injury of the cold preserved liver through the production of ROS and cytokines, like tumor necrosis factor-α (48,49). Our data suggests that high overexpression of HO-1 in Kupffer cells prior to transplantation contributes to the deleterious effects of these cells in I/R injury.

Although there is a large body of evidence suggesting that exogenous up-regulation of HO-1 in transplant models in animals confers cytoprotective effects (5,32-34), our findings caution against an uncontrolled application of non-cell specific methods to induce HO-1 expression in human organ donors. Exogenous induction of HO-1 in postmortem organ donors could further increase an already elevated HO-1 expression, resulting in potentially detrimental effects instead of cytoprotection. The main difference between our study in patients undergoing liver transplantation and studies in animal models of liver transplantation is that in the clinical situation liver grafts are usually obtained from brain death organ donors, whereas healthy animals are used as donors in experimental models. Moreover, cellular localization of HO-1 expression in human liver transplantation is predominantly restricted to the Kupffer cells, whereas in stress-exposed rat livers, HO-1 is also upregulated in hepatocytes (46).

Our data suggest a dual role for HO-1 in human liver transplants, with either cytoportection or increased cytotoxicity, depending on the initial level of overexpression. New pharmacological interventions should probably not focus on the induction of HO-1 prior to transplantation, but rather aim for induction during transplantation.

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